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Isolation and Characterization of a Microsomal Acid Retinyl Ester Hydrolase*

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Previous work demonstrated both acid and neutral, bile salt-independent retinyl ester hydrolase activities in rat liver homogenates. Here we present the purification, identification, and characterization of an acid retinyl ester hydrolase activity from solubilized rat liver microsomes. Purification to homogeneity was achieved by sequential chromatography using SP-Sepharose cation exchange, phenyl-Sepharose hydrophobic interaction, concanavalin A-Sepharose affinity and Superose 12 gel filtration chromatography. The isolated protein had a monomer molecular mass of ~62 kDa, as measured by mass spectrometry. Gel filtration chromatography of the purified protein revealed a native molecular mass of ~176 kDa, indicating that the protein exists as a homotrimeric complex in solution. The purified protein was identified as carboxylesterase ES-10 (EC 3.1.1.1) by N-terminal Edman sequencing and extensive LC-MS/MS sequence analysis and cross-reaction with an anti-ES-10 antibody. Glycosylation analysis revealed that only one of two potential *N*-linked glycosylation sites is occupied by a high mannose-type carbohydrate structure. Using retinyl palmitate in a micellar assay system the enzyme was active over a broad pH range and displayed Michaelis-Menten kinetics with a K_m of 86 μM. Substrate specificity studies showed that ES-10 is also able to catalyze hydrolysis of triolein. Cholesteryl oleate was not a substrate for ES-10 under these assay conditions. Real time reverse transcriptase-PCR and Western blot analysis revealed that ES-10 is highly expressed in liver and lung. Lower levels of ES-10 mRNA were also found in kidney, testis, and heart. A comparison of mRNA expression levels in liver demonstrated that ES-10, ES-4, and ES-3 were expressed at significantly higher levels than ES-2, an enzyme previously thought to play a major role in retinyl ester metabolism in liver. Taken together these data indicate that carboxylesterase ES-10 plays a major role in the hydrolysis of newly-endocytosed, chylomicron retinyl esters in both neutral and acidic membrane compartments of liver cells.

Higher eukaryotic organisms require dietary vitamin A (retinol), which gives rise to a variety of active metabolites. Retinoids are needed for important physiological processes such as vision, reproduction, growth, development, and immune function. Vitamin A must be obtained from the diet either as preformed vitamin A in foods of animal origin or as provitamin A carotenoids in fruits and vegetables. Preformed vitamin A is found in the diet mainly in the form of long-chain fatty acid esters (retinyl esters), with minor amounts found in the form of retinol and retinoic acid (1).

Following ingestion, dietary retinyl esters are hydrolyzed to retinol in the lumen of the small intestine. This activity was previously thought to be catalyzed by pancreatic carboxylester lipase (2); however, experiments with a carboxylester lipase knock-out mouse model indicated that the luminal hydrolysis of retinyl esters is catalyzed by pancreatic triglyceride lipase (3). Rigtrup *et al.* (4, 5) also identified an enzyme with alkaline retinyl ester hydrolase activity in intestinal brush border membranes, which is thought to be phospholipase B. Liberated retinol is subsequently absorbed by enterocytes, reesterified with long-chain fatty acids primarily by lecithin:retinol acyltransferase, incorporated into chylomicrons, and secreted into the lymphatic system.

After transport to and uptake by the liver, retinyl esters are hydrolyzed to retinol by neutral and acid retinyl ester hydrolases (REH).[1] Retinol is then transferred to the endoplasmic reticulum (ER), where it binds to retinol-binding protein (RBP) before secretion into the circulation as a retinol-RBP complex. Excess retinol is transported by a yet unknown mechanism to hepatic stellate cells for storage as retinyl ester. Mobilization of these reserves during times of inadequate dietary intake again requires hydrolysis by neutral and/or acidic REHs (1).

The enzymatic hydrolysis of retinyl esters in liver plays an important role in maintaining constant plasma retinol levels and is a key step in the formation and utilization of hepatic vitamin A reserves. Several enzymes, such as pancreatic carboxylester lipase, pancreatic triglyceride lipase, and members of the nonspecific carboxylesterase gene family have been identified in the past that can hydrolyze retinyl esters *in vitro* (6). Research has also focused on the enzyme(s) that catalyze this important reaction in liver cells. Harrison and Gad (7, 10) as well as Napoli and co-workers (8, 9, 11) showed that rat liver homogenates contain both neutral and acid bile salt-independent REH activities. The purification of different proteins from

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[1] The abbreviations used are: REH, retinyl ester hydrolase; AREH, acid retinyl ester hydrolase; AS, ammonium sulfate; Con A, concanavalin A; DSA, *Datura stramonium* agglutinin; Endo H, β-endo-*N*-acetylglucosaminidase H; MAA, *Maackia amurensis* agglutinin; MALDI-ToF, matrix-assisted laser desorption ionization-time of flight; MG, methyl-α-D-glucopyranoside; MS, mass spectrometry; NaAc, sodium acetate; OG, octyl-β-D-glucopyranoside; PNA, peanut agglutinin; PNGase F, peptide *N*-glycanase F; SELDI-TOF, surface-enhanced laser desorption ionization-time of flight; SNA, *Sambucus nigra* agglutinin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; ER, endoplasmic reticulum; RT-PCR, reverse transcriptase-PCR; RBP, retinol-binding protein.

liver with the neutral REH activity has been reported by a number of groups. Sun *et al.* (12) isolated two bile salt-independent REHs from rat liver microsomes and identified the purified proteins as rat liver carboxylesterase ES-2 (serum esterase) and ES-10 (pI 6.0/6.1 esterase). Schindler *et al.* (13) described the purification of a neutral REH from pig liver tissue and its close relationship to carboxylesterase ES-4, as judged by its structural, immunological, and catalytic features. Sanghani *et al.* (14) purified six proteins with neutral retinyl palmitate hydrolase activities from rat liver microsomal extracts, the major three of which had been previously characterized as rat liver carboxylesterses ES-10, ES-4, and ES-3. There have been no reports of the purification of acid retinyl ester hydrolases from liver or other tissues.

Retinyl ester hydrolysis in hepatocytes takes place after receptor-mediated uptake of chylomicron remnants. Hagen *et al.* (15) demonstrated in a cell culture model that most of the chylomicron remnant-derived retinyl ester is hydrolyzed in increasingly acidic endosomal compartments and that lysosomes do not contribute significantly to retinyl ester hydrolysis. Similar conclusions were drawn from experiments in intact rats by Harrison *et al.* (16). Even less is known about retinyl ester hydrolysis in hepatic stellate cells. Azais-Braesco and co-workers (17) isolated retinyl ester-containing lipid droplets from liver stellate cells and showed that an unidentified acid REH activity could use these lipid droplets as substrate for retinyl ester hydrolysis (18). The aim of the present investigation was to purify and characterize the bile salt-independent, acid REH from rat liver microsomes.

EXPERIMENTAL PROCEDURES

Materials—Frozen livers (average wet weight, ~14 g) from male Sprague-Dawley rats were obtained from Hilltop Lab Animals; retinyl-[1-^{14}C]palmitate (2.22 GBq/mmol), cholesteryl [1-^{14}C]oleate (2.22 GBq/mmol), and [9,10-^{3}H]triolein (0.74 TBq/mmol) were purchased from American Radiolabeled Chemicals; Triton X-100, Triton X-100RS, and octyl-β-D-glucopyranoside (OG) were from Sigma; Scintiverse-BD liquid scintillation liquid was from Fisher Scientific; SP-Sepharose FF, phenyl-Sepharose HP, concanavalin A-Sepharose, and Superose 12 were from Amersham Biosciences; Precision Protein Standards and Biosafe Coomassie Blue G-250 were from Bio-Rad; Peptide:*N*-glycosidase F and endoglycosidase H were from New England BioLabs; bicinchoninic acid and bovine serum albumin standards were from Pierce, Biomax 30 filters were from Millipore, H4 protein chips were from Ciphergen; and tissue-specific rat total RNA was from Ambion. All other reagents were of the highest purity available.

Isolation of Hepatic Microsomes—Rat liver microsomes were prepared as previously described by Gad and Harrison (10). Briefly, frozen rat livers were thawed and washed twice with an ice-cold sucrose/Tris buffer (250 mM sucrose, 20 mM Tris/HCl, pH 7.2). Individual livers were minced with a scalpel and processed through a tissue press. The minced liver tissue was resuspended in sucrose/Tris buffer and homogenized with three strokes of a Potter-Elvejhem homogenizer. The homogenate was centrifuged for 20 min at 10,000 × *g*. The supernatant was collected, and the pellet was resuspended in sucrose/Tris buffer, homogenized, and centrifuged again as described above. The combined supernatants were centrifuged for 60 min at 105,000 × *g*. The pelleted microsomes were resuspended with a Dounce homogenizer in sucrose buffer (250 mM) at a protein concentration of 2 mg/ml and stored in aliquots at −80 °C.

Enzyme and Protein Assays—REH activity was measured with minor modifications by a radiometric method described previously by Harrison and Gad (7). In short, 10 μl of sodium acetate buffer (250 mM, pH 5.0) and 15 μl of enzyme source (freshly thawed subcellular or eluted fractions) were added together on ice. The enzyme reaction was initiated by the addition of retinyl palmitate (500 pmol, with tracer radiolabeled retinyl-[1-^{14}C]palmitate (~12.5 nCi)) in 25 μl of water containing 0.1% (w/v) Triton X-100). Assay mixtures were incubated for 30 min at 37 °C. The reactions were terminated by the addition of 812.5 μl of methanol/chloroform/heptane (1.41:1.25:1.0, v/v/v) and 262.5 μl of potassium carbonate/borate buffer (100 mM, pH 10). An aliquot of the upper aqueous phase was mixed with scintillation solvent and counted in a liquid scintillation counter. The amount of hydrolyzed retinyl palmitate was calculated from the specific radioactivity of the substrate and the partition coefficient of palmitic acid. The same enzymatic test was applied to assay cholesteryl esterase and triolein lipase activities by substituting labeled cholesteryl oleate and labeled triolein, respectively, for retinyl palmitate as substrate. These assays were carried out with 1.5 μg of purified ES-10 (Superose 12 pool). Several different proteins are known to bind retinol. To test whether these proteins can stimulate retinyl ester hydrolase activity in our *in vitro* assay system, bovine serum albumin and β-lactoglobulin were added to the test mixture at the following concentrations: 0.01, 0.1, and 1 mg/ml. Serum retinol-binding protein and recombinant apocellular retinol-binding protein 1 were added to the test mixture at the following concentrations: 0.01 and 0.1 mg/ml. Enzyme activity measurement were carried out with 1.5 μg of purified ES-10 (Superose 12 pool) at acidic and neutral pH. Protein concentrations were quantified by the bicinchoninic acid method using bovine serum albumin as standard. All enzyme activity assays were performed three times in triplicates using ES-10 purified according to the previously described protocol. Enzyme kinetics was analyzed with the Origin 5.0 Professional software package.

Solubilization of Acid REH Activity from Hepatic Microsomal Membranes—Freshly thawed microsomes were diluted with 250 mM sucrose to a protein concentration of 2 mg/ml. Triton X-100 was added to a final concentration of 0.1% (w/v), and the mixture was incubated for 60 min at 4 °C. Following incubation, the solution was centrifuged for 60 min at 4 °C at 105,000 × *g* using a Ti 50.2 rotor (Beckman). The supernatant was collected and used as starting material for the purification of acid REH.

Purification of REH by Sequential Chromatography—Detergent-solubilized microsomes were adjusted with NaAc to pH 5.0 and a final concentration of 25 mM NaAc. The solution was clarified by centrifugation for 60 min at 105,000 × *g* and used as starting material for cation exchange chromatography. The clarified supernatant was directly applied to a SP-Sepharose FF column equilibrated with a NaAc buffer (25 mM NaAc, pH 5.0, 0.1% Triton X-100RS). After extensive washing, bound proteins were eluted with a linear gradient of 0–1 M NaCl in sodium acetate buffer. Eluted fractions were immediately neutralized with a Tris/HCl buffer to pH 7.2 and brought to a final concentration of 50 mM Tris/HCl. The fractions with the highest specific activities were pooled, adjusted to 0.5 M ammonium sulfate (AS) and loaded onto a phenyl-Sepharose column, equilibrated with an AS/Tris buffer (0.5 M AS, 50 mM Tris/HCl, pH 7.2). After extensive washing with the same buffer, bound protein was eluted with a *N*-octyl-β-D-glucopyranoside (OG)/Tris buffer (25 mM OG, 50 mM Tris/HCl, pH 7.2). Fractions with the highest specific activity were pooled, adjusted to 0.5 M NaCl, and applied to a concanavalin A-Sepharose column equilibrated with Con A buffer (30 mM Tris/HCl, pH 7.2, 500 mM NaCl, 25 mM OG, 1 mM $Mn/CaCl_2$). After extensive washing, specifically bound proteins were eluted with 0.5 M methyl-α-D-glucopyranoside in Con A buffer. Fractions with the highest specific activity were pooled, concentrated with a Biomax 30 centrifugal filter, and loaded onto a Superose 12 high resolution column equilibrated with a Tris/NaCl gel filtration buffer (20 mM Tris/HCl, pH 7.2, 150 mM NaCl, 25 mM OG). Fractions with the highest specific activity were concentrated and stored in frozen aliquots at −80 °C.

SDS-PAGE, Western Blotting, and N-terminal Sequence Analysis—SDS-PAGE analysis was carried out with the Tris/Tricine buffer system according to Schagger and Von Jagow (19). Proteins were separated on 10% SDS-PAGE minigels (10 × 7 cm) and visualized by colloidal Coomassie Blue G-250 or silver staining according to standard protocols (20, 21). SDS-PAGE-separated proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane using the Towbin transfer buffer system (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol). Transferred proteins were stained with Amido Black for 1 min, destained with diluted acetic acid for 5 min, and washed extensively with MilliQ water. N-terminal Edman sequence analysis was carried out by the Protein Microchemistry/Mass Spectrometry Facility at the Wistar Institute (Philadelphia, PA) on an Applied Biosystems Model 494 Precise Protein Sequencer. Additional sequence information was obtained by in-gel tryptic digestion of purified REH and LC-MS/MS analysis at the Protein Microchemistry/Mass Spectrometry Facility on a Finnigan LCQ Classic Ion Trap Mass Spectrometer with Nanocapillary HPLC.

On-chip Glycosylation Analysis by SELDI-TOF Mass Spectrometry—Purified REH was adjusted to 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and spotted onto the surface of an H4 protein chip pretreated with 100% acetonitrile. The sample was air-dried, and spotting was repeated two more times. After a brief wash with MilliQ water, the protein chip was placed in an 8-well bioprocessor. The samples were

TABLE I
Rat probe and primer sequences used for real time RT-PCR

Target DNA (GenBank™)	Probes (conc.)[a]	Forward primer (conc.)[a]	Reverse primer (conc.)[a]
	5′-FAM-3′-BHQ	*5′-3′*	*5′-3′*
L32 (NM_013226)	CAGCACAGCTGGCCATCAGAGTCAC (200 nM)	CCGAAAAGCCATCGTAGAAAGA (300 nM)	CCTGGCGTTGGGATTGG (900 nM)
ES-10 (NM_133295)	TGGGCTATCCACTCTCCGAAGGCA (200 nM)	AGCAAGAGTTTGGCTGGATCAT (300 nM)	AGAGGGATTTGGCTGTTTTCTG (900 nM)
ES-4 (X81825)	CTGGATGTGAAACCACCACATCTGCC (200 nM)	CAGCCGCTAAGCAAATTGCT (300 nM)	ACGAGGCAGTGAACAATGA (900 nM)
ES-3 (NM_031565)	ATCCCAATGGACAGGGCCTGCC (200 nM)	CTGGGCAAACTTTGCTAGGAA (900 nM)	TTTGGTCATACTCTGGCCAATG (900 nM)
ES-2 (NM_133586)	CACGGAAACAACCACATAGCTGGCTG (200 nM)	GCCCGACGAACTGAGAACTG (900 nM)	GAATAAGCAGGAGCCCAAAGAG (900 nM)

[a] Optimized probe and primer concentrations are indicated in parentheses.

incubated either without or with 1 unit of PNGase F (in 50 mM sodium phosphate, pH 7.5) or 1 unit of Endo H (in 50 mM sodium citrate, pH 5.5) in a final volume of 50 μl. Samples were incubated for 2 h at 37 °C. After the incubation, the protein chip was briefly washed with MilliQ water and air-dried. 1 μl of a saturated solution of sinapinnic acid in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in MilliQ water was deposited on the individual spots of the protein chips. Glycosylated and deglycosylated REH was analyzed by time-of-flight mass spectrometry.

cDNA Synthesis and Real Time RT-PCR Analysis—cDNA was synthesized in a total volume of 20 μl, from 5 μg of rat total RNA (Ambion) in the presence of random primers and ImProm-II™ reverse transcriptase (Promega), according to the manufacturer's protocol. mRNA expression was determined by real time RT-PCR on an ABI PRISM™ 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Probes and Primers (Table I) were designed with the Primer Express 1.5 software program (Applied Biosystems) and synthesized by Keystone DNA BIOSOURCE International (BIOSOURCE International, Camarillo, CA). PCR amplification was performed in capped 96-well optical plates. PCR reactions were carried out with the cDNA equivalent of 100 ng of RNA/well in a total volume of 50 μl using the Stratagene Brilliant kit (La Jolla, CA). After an initial step of 2 min at 50 °C and 10 min at 95 °C, 40 thermal cycles were carried out for 15 s at 95 °C and 1 min at 60 °C. Relative quantification of gene expression was performed by the Comparative Threshold Cycle (C_T) method (ABI PRISM 7700 Sequence Detection System, User Bulletin 2) using the rat ribosomal protein L32 as standard for comparisons.

RESULTS

Purification of Acid Retinyl Ester Hydrolase—Gad and Harrison (10) have reported previously that rat liver microsomes contain both bile salt-independent, neutral and bile salt-independent, acid REH activities. Rat liver microsomes were therefore used as enzyme source for developing an AREH purification protocol. Initially, a number of different non-ionic detergents were screened for their ability to effectively solubilize AREH activity from microsomal membranes. Triton X-100, Nonidet P40, Tween 20, OG, and OTG were found to be equally effective solubilizers of AREH activity when used above their respective critical micellar concentrations (data not shown). AREH activity was subsequently purified from Triton X-100-solubilized microsomal membranes by sequential chromatography using cation ion exchange, hydrophobic interaction and lectin affinity chromatography. Trial experiments showed that binding of AREH activity to concanavalin A-Sepharose was inhibited by the presence of Triton X-100 in the binding buffer; however, increased affinity of AREH activity to Con A-Sepharose was observed when Triton X-100 was replaced by OG in the binding buffer. As a consequence, a detergent exchange was incorporated into the elution protocol of the hydrophobic interaction chromatography step. Purification to near homogeneity was achieved by a final gel filtration step with a calibrated Superose 12 HR10/30 column, which not only removed remaining impurities but also provided additional insight into the degree of complexation of AREH activity in solution. Analytical gel filtration estimated the native molecular mass of AREH to be ~176 kDa (Fig. 1). The presence of octyl glucoside in the gel filtration buffer was necessary to prevent a significant loss of total retinyl ester hydrolase activity during this purification step. The purification of AREH activity was monitored by the increase in specific activity (Table II) and by SDS-PAGE (Fig. 2). Fig. 2 shows that the enrichment of AREH activity resulted in the purification of a protein with a monomer molecular mass of ~60 kDa as judged by SDS-PAGE under reducing conditions. Taken together with the analytical gel filtration results, these data indicate that AREH activity exists as a homotrimeric complex in solution. Densitometric analysis of the gel filtration eluate revealed that the AREH-containing sample was ~95% pure (Fig. 2, *lanes 6* and *7*). Table II summarizes the purification of AREH activity using 5 rat livers as starting material. To date, 4 preparations of the enzyme have been carried out according to the described protocol with similar results in specific activity, yield and enrichment to those shown in Table II. Table II demonstrates that Triton X-100 not only releases AREH from microsomal membranes but that it also stimulates total AREH activity ~3-fold. Enrichment and yield of AREH activity were therefore calculated in respect to the solubilized supernatant of the microsomal membrane preparation to take this stimulatory effect into account. In contrast, AREH activity became increasingly unstable once it was solubilized from microsomal membranes, which resulted in a loss of over 90% of total AREH activity during the subsequent four chromatographic purification steps (Table II). Because the sensitivity of AREH to serine and cysteine protease inhibitors was not known, these inhibitors were not included in the solubilization buffer. Furthermore, some proteasomes are associated with microsomal membranes (22) and might therefore be responsible for the loss of enzymatic activity observed after the solubilization and subsequent purification steps.

Identification of AREH as Carboxylesterase ES-10—In order to identify the AREH activity, the purified protein was subjected to in-gel tryptic digestion, followed by MALDI-TOF analysis of the resulting peptides. The resulting peptide mass fingerprint was then compared with the peptide mass fingerprints of all known rat proteins in the non-redundant NCBI data base with the Mascot protein search software. The initial search successfully matched the tryptic peptide map to several different proteins of the highly homologous carboxylesterase family but failed to unambiguously identify a single protein. As a consequence, the in-gel tryptic digest of the purified protein was subjected to LC-MS/MS sequence analysis, which identified the purified AREH activity as rat liver carboxylesterase ES-10. The N terminus of mature ES-10 was identified by automated Edman degradation of the intact protein and was found to have the sequence: YPSSPPVVNTVKGKV (Table III). A polyclonal antibody raised against the 13 C-terminal amino acids of ES-10 also reacted with the purified protein, further confirming the identification of AREH as ES-10 (data not shown).

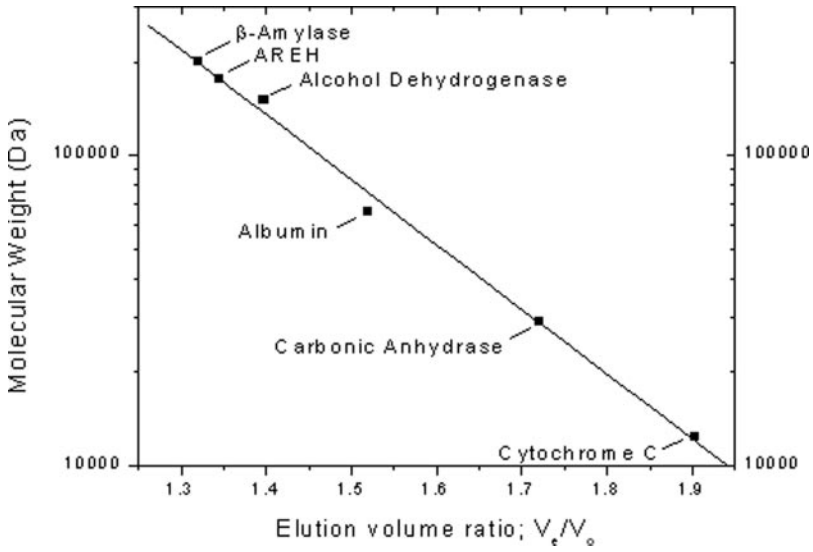

FIG. 1. **Gel filtration analysis of purified rat liver AREH.** Pooled and concentrated fractions of the Con A column were applied to a Superose 12 HR10/30 gel filtration column and eluted isocratically as described under "Experimental Procedures." Fig. 1 shows the calibration curve of the Superose 12 HR10/30 column. Molecular mass standards used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

TABLE II
Purification of a bile salt-independent AREH from solubilized rat liver microsomes

Fraction	Volume	Protein. conc.	Total protein	Total activity	Specific activity	Enrichment	Yield
	ml	*mg/ml*	*mg*	*nmol/h*	*nmol/mg·h*	*fold*	*%*
Microsomes	50	12.8	640	704	1.1		
Solubilized microsomal supernatant	320	0.5	160	2080	13	1	100
SP-Sepharose pool	50	0.6	30	897	29.9	2.3	43
Phenyl-Sepharose pool	20	0.3	6	247	41.3	3.2	11.8
Con A-Sepharose pool	18	0.18	3.2	203	63.5	4.9	9.7
Superose 12 gel filtration	8	0.32	2.56	172	67.4	5.2	8.3

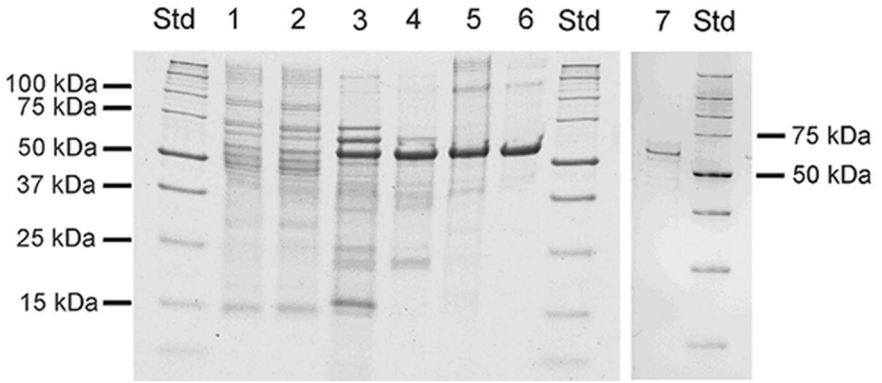

FIG. 2. **SDS-PAGE analysis of AREH during protein purification.** Approximately 2.5–3 μg of total protein were applied to each lane and separated under reducing conditions on a 10% Tris-Tricine SDS-PAGE gel. Proteins were visualized by colloidal Coomassie Blue staining or silver staining. *Lane 1*, microsomes; *lane 2*, solubilized microsomes; *lane 3*, SP-Sepharose pool; *lane 4*, phenyl-Sepharose pool; *lane 5*, Con A-Sepharose pool; *lane 6*, Superose 12 pool; *lane 7*, silver-stained Superose 12 pool.

TABLE III
Enzymatic properties of carboxylesterase ES-10 as a bile salt-independent REH

Molecular mass (kDa)[a]	
Glycosylated ES-10	62
Deglycosylated ES-10[b]	60.3
Carbohydrate structure of *N*-glycan	High mannose-type
N-terminal amino acid	Tyr^{19}
K_m (μM)[c]	86 ± 12 (n = 3)
*V*max (nmol h^{-1} mg^{-1})[c]	724 ± 48 (n = 3)
Specific activity (nmol h^{-1} mg^{-1})[c,d]	437

[a] Determined by MALDI-ToF MS.
[b] After PNGase F treatment.
[c] At pH 5.0.
[d] At a substrate concentration of 100 μM.

Enzymatic Deglycosylation of ES-10—The analysis of the amino acid sequence of ES-10 revealed two potential *N*-linked glycosylation sites at positions 79 and 489. In order to analyze the glycosylation of ES-10 in more detail, the purified protein was spotted onto the surface of a NP20 protein chip, incubated with either PNGase F or Endo H and analyzed by SELDI-TOF mass spectrometry. Deglycosylation of purified ES-10 with PNGase F reduced the molecular mass from 62.0 kDa (Fig. 3*A*) to 60.3 kDa (Fig. 3*B*) whereas the deglycosylation of ES-10 with Endo H reduced the mass to ~60.4 kDa (Fig. 3*C*). In addition, the carbohydrate structure of ES-10 was probed with a number of different digoxigenin-labeled lectins that selectively recognize specific terminal sugars and thus distinguish between high mannose, complex and hybrid *N*-glycans, and *O*-glycosidic-linked carbohydrate structures. Of all lectins tested, ES-10 cross-reacted only with GNA, which recognizes terminal mannose residues of high mannose *N*-glycans and has a similar binding specificity as Con A. None of the other lectins (SNA, MAA, PNA, and DSA) tested were bound by the carbohydrate structure of ES-10 (data not shown).

Enzymatic Characterization of ES-10 as a Retinyl Ester Hydrolase—The enzymatic activity of purified rat liver carboxylesterase ES-10 was measured as a function of pH in the range of 2.5 to 10. Fig. 4*A* shows that ES-10 exhibited optimal activity at pH 8.0; however, it is noteworthy that ES-10 retained 80–90% of its maximal activity in the physiologically relevant pH

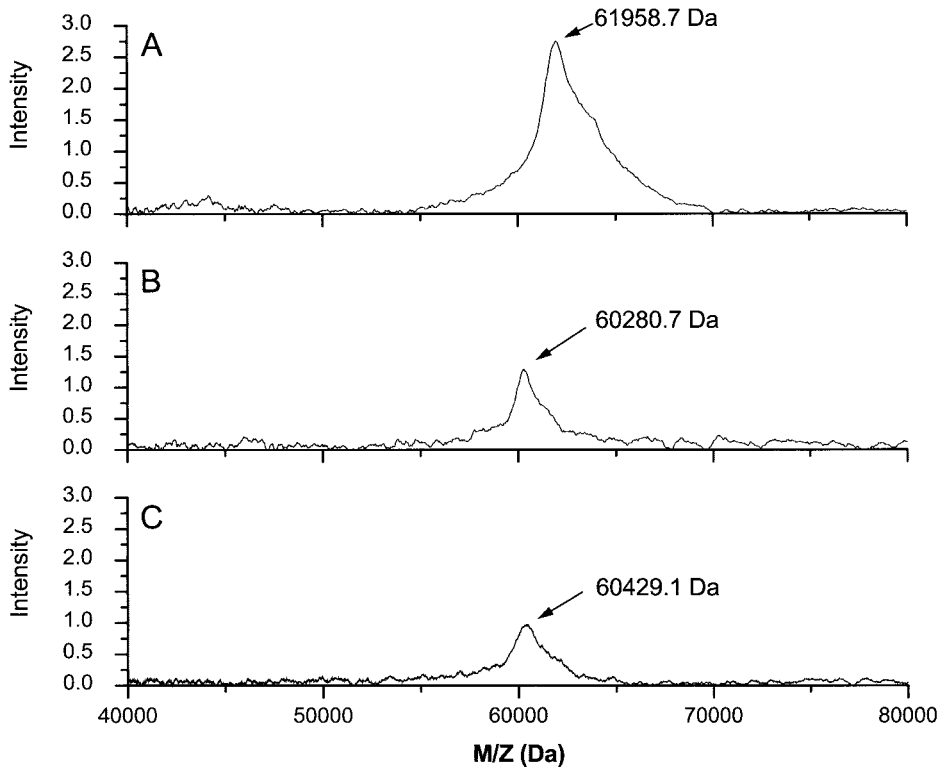

FIG. 3. **Deglycosylation of purified ES-10.** Purified ES-10 was incubated on the surface of an H4 chip with either PNGase F or Endo H, and then subsequently analyzed by SELDI-TOF MS as described under "Experimental Procedures." *A*, untreated ES-10; *B*, purified ES-10 after treatment with PNGase F; *C*, purified ES-10 after treatment with Endo H.

range of 7.4–5.0 and that ES-10 had significant enzymatic activity down to pH 4. K_m and V_{max} were determined by increasing the substrate concentration in the assay while keeping the amount of detergent used to solubilize the substrate constant. Fig. 4*B* shows that ES-10 displayed Michaelis Menten kinetics under the chosen assay conditions and that the rate of hydrolysis increased in a linear fashion up to a substrate concentration of 50 μM. Maximal rates of hydrolysis were observed at substrate concentrations in excess of 100 μM. The observed specific activities were analyzed by non-linear curve fitting to a one-site binding model. Under the chosen assay conditions, ES-10 had a K_m of 86 μM and a V_{max} of 742 nmol/h·mg (Table III). Several different lipid substrates were tested in the micellar assay system, but only retinyl palmitate was found to be an efficient substrate for ES-10, whereas no activity was detected with cholesteryl oleate. Triolein was a substrate for ES-10, although at a much slower rate (Fig. 4*C*). Additional experiments were carried out to determine whether retinol-binding proteins had any effect on the hydrolytic activity of ES-10. None of the proteins tested, including plasma RBP, recombinant apo-CRBP1, β-lactoglobulin, and albumin increased the rate of retinyl ester hydrolysis in the micellar assay system (data not shown). The addition of physiological levels of bile salts to the assay mixture also had no significant effect on the rate of retinyl palmitate hydrolysis by purified ES-10 (data not shown).

Tissue Specificity of the ES-10 Gene and Protein Expression—Tissue-specific primers and probes (Table I) were designed to measure the relative gene expression of ES-10 in various tissues by real time RT-PCR. Fig. 5 shows that ES-10 mRNA is highly expressed in liver and that comparable levels of ES-10 mRNA are also found in the lung as well. Kidney, testis, and heart expressed only about 10–15% of the level of ES-10 mRNA in liver, whereas the expression of ES-10 in brain, ovary, and intestine was less than 3% that of liver. A number of tissues were probed with an anti-ES-10 antibody to complement the gene expression experiments. Western blot analysis revealed high levels of ES-10 protein expression in liver, lung, and testis, whereas the expression of ES-10 protein in kidney and heart was not detected with this antibody (Fig. 6) possibly because of the C-terminal specificity of this antibody. Additional probes and primers were designed to compare the gene expression of different carboxylesterases in rat liver. Fig. 7 shows that the expression of ES-10 in liver was significantly higher than that of ES-2, an enzyme previously thought to play a major role in liver retinyl ester metabolism (12).

DISCUSSION

The enzymatic hydrolysis of retinyl esters in liver plays an important role in maintaining retinol homeostasis and is a key step in the formation and mobilization of hepatic vitamin A reserves. Hepatic vitamin A metabolism begins with the uptake of retinyl ester-containing chylomicron remnants by low density lipoprotein receptor-mediated endocytosis (23). Blomhoff *et al.* (24) demonstrated in a previous study by subcellular fractionation in density gradients that endocytosed chylomicron remnant [^{3}H]retinyl esters and ^{125}I-asialofetuin are initially located in low density endosomes of rat liver parenchymal cells. However, unlike ^{125}I-asioalofetuin, which was subsequently transported to and degraded in late endosomal/lysosomal compartments, radioactively labeled retinoid was probably transferred to the endoplasmic reticulum (ER), as indicated by the co-migration of [^{3}H]retinoid and the ER marker enzymes glucose-6-phosphatase and rotenone-insensitive NADPH-cytochrome *c* reductase. These studies were later extended by Harrison *et al.* (16) who showed that newly delivered, radioactively labeled retinyl ester co-localized with bile salt-independent, neutral, and acid retinyl ester hydrolase activities in enriched plasma membrane/endosomal fractions. Despite the details provided by these studies, two intriguing questions remain largely unanswered. (*a*) Does retinyl ester hydrolysis occur in rat liver parenchymal cells before or after retinoid transport to the ER? (*b*) How is retinol/retinyl ester selectively transferred to the ER while cholesteryl esters and triacylglycerols derived from endocytosed lipoproteins are transported to lysosomes for degradation (25)? The goal of this

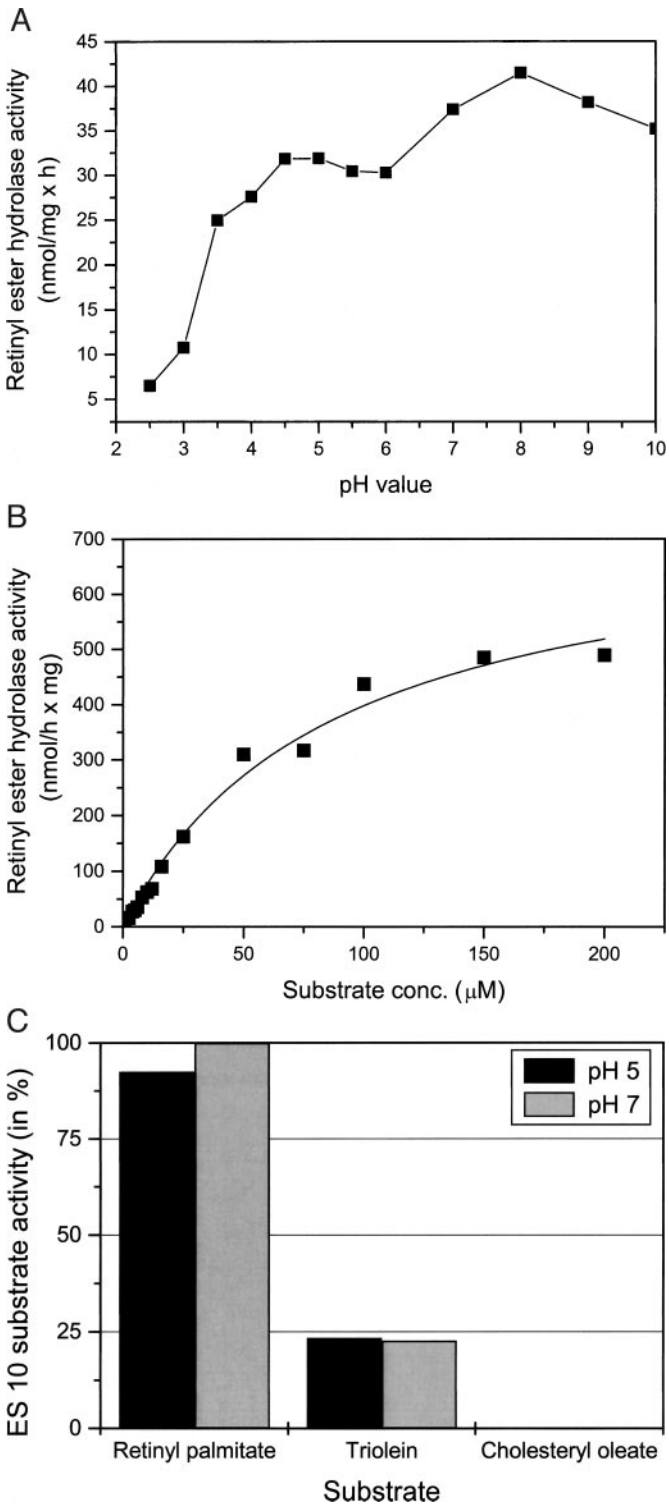

FIG. 4. **Enzymatic characterization of purified ES-10 as a retinyl ester hydrolase.** *A*, effect of pH on purified REH activity. Assays were conducted with 50 mM buffers at the indicated pH values. *B*, effect of substrate concentration on AREH activity. Assays were conducted in the presence of rising substrate concentrations while keeping the concentration of Triton X-100 constant. *C*, substrate specificity of ES-10 in a micellar assay system at a substrate concentration of 100 μM at the indicated pH value.

study was therefore to purify the enzymes that are able to hydrolyze retinyl esters in the increasingly acidic environment of the endocytotic pathway. The identity of these proteins might then provide important new clues to the possible localization of retinyl ester hydrolysis in hepatocytes.

Our approach to the purification of the bile salt-independent acid retinyl ester hydrolase was to monitor the enrichment in specific activity at pH 5.0 and to exclude bile salts from the *in vitro* assay mixture. The purified protein was subsequently identified as carboxylesterase-10 (also referred to as pI 6.0/6.1 esterase) by N-terminal Edman sequencing of the intact protein and a combination of MALDI-TOF and LC-/MS/MS sequence analysis of tryptic peptides. These results were further supported by the observation that the purified acid retinyl ester hydrolase cross-reacted with a polyclonal anti-ES-10 antibody directed at the C terminus of ES-10. Analytical gel filtration (Fig. 1) showed that the active protein exists as a homotrimeric complex in solution, as had been described for ES-10 previously (26, 27). ES-10 is a member of the nonspecific carboxylesterase supergene family (28) and is able to hydrolyze both xenobiotic and lipid substrates (27). The ability of ES-10 to hydrolyze retinyl ester at neutral pH in the absence of bile salts or bile salt derivatives has been reported previously (12) and is supported by our own results (Fig. 4*A*). These data demonstrate that ES-10 has the catalytic properties that would enable it to function both in the neutral and acidic endosomal environments. The addition of recombinant apo-CRBP1 to the micellar assay system did not result in an increased rate of retinyl ester hydrolysis by purified ES-10. In contrast, Boerman and Napoli (11) described the stimulation of cholate-independent retinyl ester hydrolysis by apo-CRBP1 of endogenous retinyl esters of rat liver microsomes. These two opposing observations are possibly caused by the different assay methodologies used to measure retinyl ester hydrolase activity: (*a*) micellar, detergent-solubilized retinyl palmitate *versus* phospholipid-bound, endogenous retinyl esters and (*b*) highly purified ES-10 *versus* microsomes. These differences indicate that the stimulatory effect was highly dependent on the presence of phospholipid bilayer membranes in the assay mixture (29). The strong stimulatory effect of apo-CRBP1 on retinyl ester hydrolysis was not observed at higher cholate concentrations (>8 mM, cmc of cholate: ~7–8 mM at pH 8), probably because of the solubilization of microsomes into cholate-phospholipid micelles. The same authors speculated that the stimulatory effect of apo-CRBP1 on retinyl ester hydrolysis is possibly caused by a conformational change of retinyl ester hydrolase as a result of a direct protein-protein interaction between apo-CRBP1 and retinyl ester hydrolase (11). Whereas we cannot rule out that the detergents used in our test system possibly interfered with such a suggested interaction, we would point out that CRBP1 is a cytosolic protein, whereas ES-10 is most likely located in the lumen of the endoplasmic reticulum and/or endosomes. The different subcellular locations of these two proteins would therefore preclude such an interaction. In addition, two recent studies of retinol turnover in liver of wild-type and in CRBP1 knock-out mice also suggest that CRBP1 facilitates retinyl ester synthesis by LRAT rather than hydrolysis by REH and that CRBP1 might play an important role in retinol transport between hepatocytes and stellate cells (30, 31). The observed differences between our report and the previous report illustrate the need for additional research to define the intracellular protein-protein interactions of both CRBP1 and retinyl ester hydrolase in greater detail. The recent development of novel protein-protein interaction screens in conjunction with the availability of recombinant CRBP1 should facilitate this research.

To date, four different proteins of the nonspecific carboxylesterase supergene family have been identified that are able to hydrolyze retinyl esters *in vitro*: ES-2, ES-3, ES-4, and ES-10,

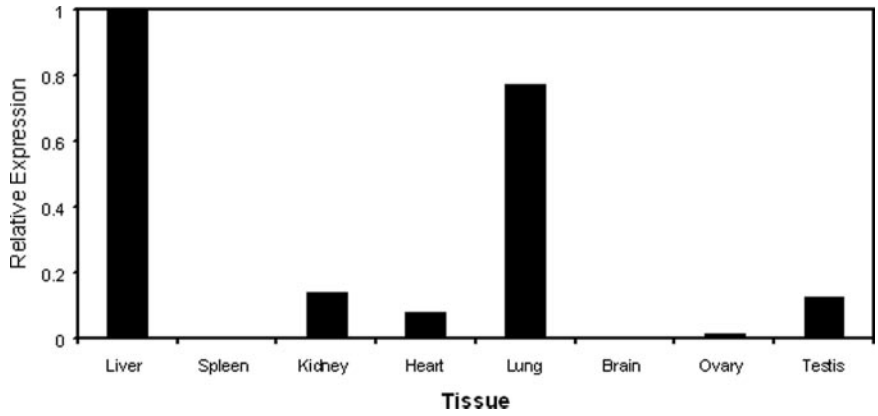

FIG. 5. **Tissue specificity of ES-10 gene expression.** ES-10 gene expression in different tissues was compared with the relative threshold cycle method using rat L32 as a housekeeping gene. For relative gene quantification, the level of ES-10 gene expression was set to 1.

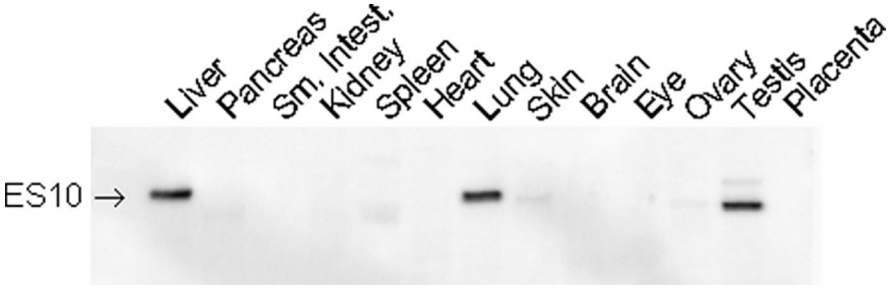

FIG. 6. **Tissue specificity of ES-10 protein expression.** Total tissue protein extracts were separated on a 10% Tris-Tricine SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes. ES-10 was detected with an anti-ES-10 antibody and visualized with the Super-Signal West Dura Extended Duration Substrate kit (Pierce).

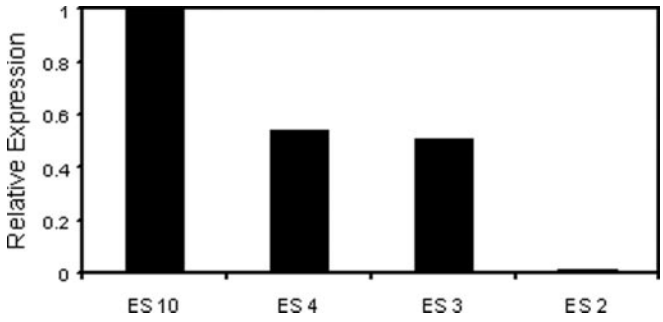

FIG. 7. **Relative gene expression of ES-2, ES-3, ES-4, and ES-10.** The gene expression of different carboxylesterases in rat liver was compared with the relative threshold cycle method using rat L32 as the housekeeping gene. For relative gene quantification the level of ES-10 expression was set to 1.

(12–14, 32). Sequence analysis of these proteins reveals that ES-3, ES-4, and ES-10 share the tetrapeptide sequence, H*X*EL, at their respective C termini. The sequence H*X*EL is a variation of the eukaryotic ER retention signal, KDEL, and has been shown to be an efficient retention sequence that localizes carboxylesterases, including ES-10, to the lumen of the ER (33). In contrast, ES-2 (also called serum carboxylesterase) lacks this C-terminal consensus sequence and probably represents a secretory form of the carboxylesterases (34).

The glycosylation pattern, more specifically the extent and complexity of glycosylation, can be used to gain additional insight into the subcellular localization and intracellular transport of glycoproteins. Because we did not obtain direct sequence information of the ES-10 C terminus by LC-MS/MS analysis we investigated the glycosylation pattern of ES-10 in more detail. ES-10 has two potential *N*-glycosylation sites at residues Asn^{79} and Asn^{479} and is sensitive to deglycosylation with PNGase F and Endo H (Fig. 3). Deglycosylation with PNGase F and Endo H reduced the molecular mass of the purified protein from 62 kDa to ~60.3 and 60.4 kDa, respectively, in good agreement with the theoretical average protein molecular mass of 60.05 kDa for mature ES-10. The substrate specificity of PNGase F and Endo H suggests that ES-10 contains high mannose-type carbohydrate structures (35). The molecular mass difference of less than 2 kDa between the glycosylated and deglycosylated form of mature ES-10 suggests that only one of two *N*-linked glycosylation sites in ES-10 is used and that the carbohydrate structure is most likely of the high mannose type. This is further supported by our observation that ES-10 can be purified with immobilized Con A-Sepharose, which is known to bind Asn-linked oligosaccharides of the biantennary complex-type and high mannose-type, but not complex triantennary and tetra-antennary carbohydrate structures (36). Probing of purified ES-10 with several different lectins such as SNA, MAA, PNA, and DSA that recognize sialic acid, galactose β-(1–3)-*N*-acetylgalactosamine, and Galβ-(1–4)-GlcNAc carbohydrate structures, respectively also failed to detect a more complex glycosylation pattern. The absence of a more complex glycosylation pattern indicates that ES-10 does not traverse the Golgi compartment and is indeed retained in the ER. In contrast, serum carboxylesterase (ES-2) is resistant to deglycosylation with Endo H, and glycosylation contributes as much as 20% to the apparent molecular weight of this secreted protein (34).

The relative contribution of the different carboxylesterases to the overall hepatic retinyl ester turnover is difficult to assess because of the variety of assay conditions used to measure the specific enzymatic activities. Sanghani *et al.* (14) recently estimated that ES-10 is responsible for ~60% and ES-4 for ~34% of the total neutral retinyl ester hydrolysis activity in rat liver microsomes, as judged by the catalytic efficiency of these two enzymes in the presence of high concentrations of bile salts. However, it remains unclear whether ES-4 does indeed contribute to a large extent to hepatic retinyl ester turnover. Two recent reports demonstrated that acyl-CoA thioesters are the preferred lipid substrates for ES-4 (37, 38), and there are conflicting reports about the ability of ES-4 to hydrolyze retinyl esters in the absence of bile salts (13, 28). In contrast, ES-10 showed a substrate preference for retinyl esters over triglycerides in a bile salt-free assay system, with no activity toward cholesteryl esters (Fig. 4*C*). These observations point toward ES-10 as the most prominent retinyl ester hydrolase in rat liver, especially if one also takes the relative levels of gene expression of ES-10 into account (Fig. 6). The special role of

ES-10 in hepatic retinyl ester metabolism is further underscored by a recent proteomic analysis of rat hepatic stellate cells in which ES-10 was among the 150 stellate cell proteins identified and the only protein with known retinyl ester hydrolase activity (39).

The role that ES-10 plays in retinyl ester metabolism will most likely depend on the subcellular localization of this reaction. If retinyl esters are transported to the ER before hydrolysis, they will most likely be hydrolyzed by ES-10 and, perhaps to a lesser degree, by ES-4. This takes into account that ES-10 and ES-4 are predominantly localized in the ER and that they contribute most of the total carboxylesterase activity detected in the ER. On the other hand, if retinyl esters remain in early endosomes they could also be hydrolyzed by ES-2, which has been localized in plasma membrane/endosomal fractions (12). It is conceivable that ES-2 undergoes a recycling process that localizes it to early endosomal compartments after endocytotic uptake alongside chylomicron remnants. A similar recycling process has been described recently for ApoE (40). The identification of the relevant retinyl ester hydrolysis pathway will require more detailed information about (*a*) intracellular retinol trafficking in hepatocytes and (*b*) the subcellular localization of ES-2, ES-3, ES-4, and ES-10. Progress in this area has been hampered because of the lack of specific antibodies that can differentiate between the highly related carboxylesterases. However, with the recent development of gene silencing by RNA interference to study protein function, we now have a tool that should help us to address these important questions.

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